

In vivo T-cell ablation by a holo-immunotoxin directed at human CD3

(diphtheria toxin/receptor/immunosuppression)

DAVID M. NEVILLE, JR.*[†], JOSHUA SCHARFF, AND KASTURI SRINIVASACHAR

Section on Biophysical Chemistry, Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, MD 20892

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ABSTRACT We have evaluated the *in vivo* efficacy of anti-CD3–CRM9, a holo-immunotoxin constructed with a diphtheria toxin binding-site mutant. Eighty percent of established human T-cell subcutaneous tumors in nude mice completely regressed following intraperitoneal injection of immunotoxin at a dose set at half the minimum lethal dose assayed in toxin-sensitive animals. Similar regressions produced by a ^{137}Cs source required a dose in excess of 500 cGy. The high degree of *in vivo* T-cell ablation produced by this immunotoxin is apparently due to maintenance of the toxin translocation function provided by CRM9 and a necessary intracellular routing function supplied by CD3. This immunotoxin may be useful in treating conditions caused by pathologic oligoclonal T-cell expansion such as graft-versus-host disease, autoimmune diseases, and possibly AIDS.

The protein toxins, of which diphtheria toxin (DT) may be considered the prototype, have their effector or enzymatic functions on domains separate from the receptor-mediated entry functions (1–4). By mixing and splicing toxin domains with binding domains derived from growth factors or monoclonal antibodies, a wide variety of immunotoxins, toxins with altered cellular specificity, have been created (5, 6). The goal has been to maximize *in vivo* targeted cell killing for therapeutic and experimental purposes (5).

Recently, Youle and coworkers (7–9) have introduced highly efficacious holoimmunotoxins based on DT binding mutants. These immunotoxins were equal in potency to immunotoxins made with wild-type DT when directed at the human transferrin receptor or a component of the human T-cell receptor complex, CD3. Because the binding of the mutants was only 1/100th to 1/1000th that of native DT, non-target cell toxicity was similarly reduced. We showed that intracellular routing of DT-based immunotoxins via intracellular DT receptors or alternative receptors is an important determinant of their efficacy (10). CD3 and the transferrin receptor apparently are routed along the same path as the DT receptor. Consequently, CRM9 immunotoxins, which lack the DT binding site, are routed appropriately when directed at CD3 and the transferrin receptor (10). Because CD3 is highly restricted to mature T cells, immunotoxins directed toward this epitope should be useful in producing pan-T-cell ablation *in vivo*. We have evaluated anti-CD3–CRM9 for this purpose.

MATERIALS AND METHODS

Immunotoxins. Anti-CD3 and anti-CD5 immunotoxins were compared in this study. CRM9 is a DT binding-site mutant that exhibits 1/1000th the binding affinity of DT (11). The biological properties of CRM9 are similar to those of

CRM107 (7, 10). CRM107 was originally reported to contain a double mutation (7) but contains only a mutation of serine to phenylalanine within the C-terminal region, at position 525 (R. J. Youle and P. J. Nicholls, personal communication). The strain of *Corynebacterium diphtheriae* used for production of CRM9, C7 ($b^h \text{tax-201 tax-9 h}^+$) was obtained from R. Holmes (Uniformed Services University of the Health Sciences, Bethesda, MD). This strain contains the β phage mutant 201, which permits increased amounts of toxin to be produced by infected cells under high-iron conditions, thus simplifying toxin production (12). Immunotoxins were synthesized as previously described (10), by thiolating both the monoclonal antibody moiety and the toxin moiety and then crosslinking with bismaleimide crosslinkers. Purification was performed by size-exclusion HPLC, and fractions containing 1:1 toxin/antibody molar ratios were isolated for these studies. Conjugates made with an acid-labile crosslinker, 1,3-bis(maleimidoethoxy)propane, were compared with a non-cleavable crosslinker, 1,6-bis(maleimido)hexane. Conjugates made with this cleavable crosslinker are hydrolyzed within the acidifying endosome, releasing free toxin moieties with half-times of hydrolysis of 36 min measured at pH 5.5 (10).

Athymic Mouse Xenograft System. N:NIH/bg/nu/xid mice (Charles River Breeding Laboratories), deficient in killer cell immune response (13), were maintained in a semisterile environment and were preconditioned with 400 cGy of whole-body ^{137}Cs γ radiation on day –7. On day 0, 2.5×10^7 Jurkat cells (human T-cell leukemia, CD3⁺CD4⁺CD5⁺) were injected subcutaneously along with 10^7 HT-1080 feeder cells (human sarcoma) that had received 6000 cGy (14). By day 7, the tumor was visibly established and could be seen as a small subcutaneous nodule ($3 \times 3 \times 3$ –5 mm) at the injection site (Fig. 1B). Peeling back the skin flap revealed a red, highly vascularized tumor nodule weighing, on average, 20 mg (Fig. 1A). CRM9-based immunotoxin treatment was given by intraperitoneal injection starting on day 7 and was set at half the minimum lethal dose (MLD) in guinea pigs. (Guinea pigs, like humans, are highly sensitive to DT. Mice are highly resistant to DT and exhibit equal sensitivity to wild-type toxin and CRM9). Mice were initially evaluated for visible tumor regression at day 14. About 10% of evaluations at day 14 were subject to change. Final evaluations were done on day 37 and these remained unchanged over the course of the study (56 days). Regression was defined by the absence of visible tumor at the injection site. Mice that failed to reveal a subcutaneous tumor nodule were randomly subjected to autopsy. No trace of a vascularized nodule as seen in Fig. 1A was ever found, nor was residual scarring present. MLD was defined as the minimum tested dose that resulted in both nonsurvivors and survivors over a 4-week evaluation period. All animals survived a dose equal to half the MLD. MLD was evaluated in guinea pigs (300–1000 g) by subcutaneous injection. The following MLDs were found (listed as μg of toxin

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Abbreviations: DT, diphtheria toxin; MLD, minimum lethal dose.
*To whom reprint requests should be addressed.

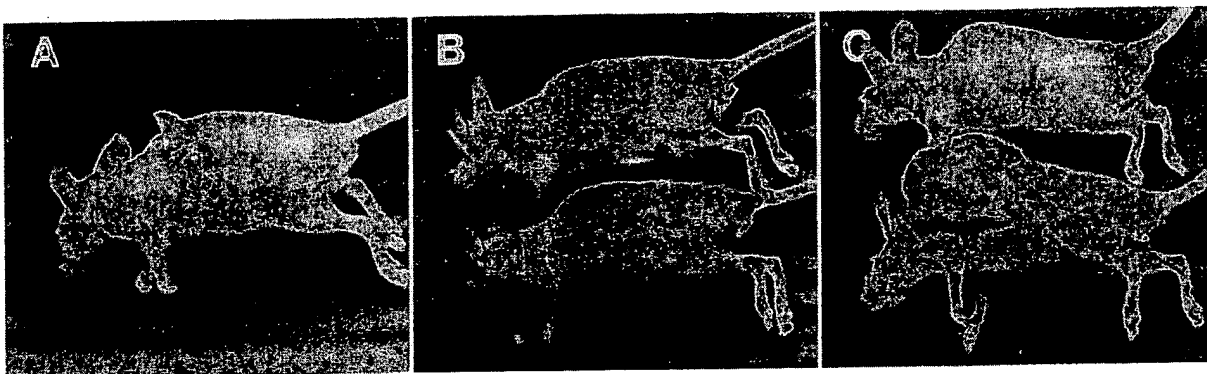


FIG. 1. Athymic mice bearing established Jurkat cell tumors. (A) Tumor at 7 days, the day of treatment. The skin over the subcutaneous tumor has been peeled back to reveal a bright red tumor nodule. No remnants of this structure were seen in mice undergoing treatment-induced regressions at day 37. (B) Same as A but without excision of the skin flap. The subcutaneous nodule is clearly visible just behind the left shoulder. (C) Tumor growth at day 37, the treatment evaluation day.

per kg of body weight): DT, 0.15; CRM9, 30; anti-CD5-DT (cleavable), 0.65; anti-CD5-CRM9 (noncleavable), 150. Jurkat cells, which have a normal complement of CD3 receptors (13), were passaged every other week in mice as subcutaneous tumors and were dissociated by collagenase/dispase fortified with an equal increment of collagenase D (Boehringer Mannheim) prior to inoculation. This cell population exhibits a 40% inhibition of protein synthesis after 5 hr of exposure to 10 pM anti-CD3-DT. Clones isolated from this population by infinite dilution exhibited varying sensitivity to anti-CD3-DT (four less sensitive, three more sensitive) corresponding to a 1.5-log variation in dose-response curves. The uncloned cell stock was used to provide a more realistic tumor model exhibiting a spectrum of immunotoxin sensitivities.

Statistical Analysis of Data. Statistical significance was determined by calculating the probability that the tumor regressions found in a treatment group occurred by the chance selection of mice with spontaneously regressing tumors into the treatment group. We did not note any regressions among the 24 controls in Table 1, and we never noted a spontaneous regression in an untreated mouse after a tumor reached a visible size (20 mg). To calculate the probability of selecting spontaneously regressing animals into a treatment group, we must know the proportion of these animals (p) in our tumor-bearing population. We may set an upper bound on this by assuming that if we had looked at one more untreated or control animal we would have found a spontaneous regression. Therefore, we will set $p = 1/(24 + 1) = 0.04$ (actually $p \leq 0.022$, since we followed another 20 untreated tumor-bearing animals not listed in Table 1 and failed to observe a spontaneous regression up to day 56).

Using the binomial distribution we calculate $P(x)$, the probability of x animals being in category p for random sample size n where $q = 1 - p$.

$$P(x) = \frac{n! p^x q^{n-x}}{x!(n-x)!}$$

For treatment group 1, $n = 6$, $x = 5$, $p = 0.04$,

$$\begin{aligned} P(x) &= \frac{6!(0.04)^5(0.96)^{6-5}}{5!(6-5)!} \\ &= (6)(0.98 \times 10^{-7}) = 5.9 \times 10^{-7}. \end{aligned}$$

The probabilities for the more extreme outcomes where x assumes values up to n have also been calculated and added

to $P(x)$, giving $P'(x)$. The values of $P'(x)$ for all important treatment groups are listed in the far right column of Table 1.

Evaluation of Tumor Cell Killing. The therapeutic equivalence of total body radiation and immunotoxin anti-CD3-CRM9 was assessed by determining the dose from a calibrated ^{137}Cs source (102 cGy/min) that produced the same frequency of complete tumor regressions, defined here as no visible recurrence over the course of the study, 56 days. This dose, determined to be 600 cGy, was given to three animals bearing $3 \times 3 \times 5$ -mm tumors. Eighteen hours later the tumors were pooled, and the cells were dispersed by collagenase/dispase, freed from debris by centrifugation, and counted. Cells were serially diluted in a 96-well plate in RPMI conditioned medium containing 10% fetal bovine serum. Plating efficiency, the reciprocal of the minimum average number of cells per well that will grow to form one colony, was determined after 6 weeks and compared with that for a nonirradiated tumor similarly processed.

RESULTS

Subcutaneous vascularized human T-cell tumors (Jurkat, $\text{CD3}^+\text{CD4}^+\text{CD5}^+$) were established in conditioned nude mice. Intraperitoneal treatment with immunotoxin was begun after 7 days as illustrated in Fig. 2. The results are tabulated in Table 1. Groups that did not show regression comprised 24 mice and included the untreated group (group 10), groups treated with anti-CD5 immunotoxins (groups 5 and 6), and a group treated with a mixture of anti-CD3 and CRM9 (group 4). The vascularized tumor nodules, which averaged 20 mg on day 7, grew to between 1.5 and 7.8 g on day 37 (see Fig. 1C) and to between 7.9 and 11.6 g on day 56. No late spontaneous regressions were noted. In contrast, group 1, consisting of mice given anti-CD3-CRM9 noncleavable conjugate at 25 $\mu\text{g}/\text{kg}$ on days 7, 8, and 9 (see Fig. 2 time line), showed only 1 tumor out of 6 by day 37. Adding anti-CD5-CRM9 cleavable conjugate with anti-CD3-CRM9 (group 2) failed to potentiate efficacy beyond that observed in group 1. Anti-CD3-CRM9 treatment groups 1 and 2 exhibited 8 regressions out of 10 animals, in marked contrast to the control groups and the anti-CD5 immunotoxin groups, which failed to show any regressions for 24 animals. When some of the mice exhibiting regressions were subjected to autopsy, no residual tumor or even scarring was seen. Tumors identified as regressed on day 37 by superficial inspection did not reappear during the course of the study (56 days).

Results with immunotoxins constructed with a noncleavable crosslinker and directed at CD3 and CD5 were consis-

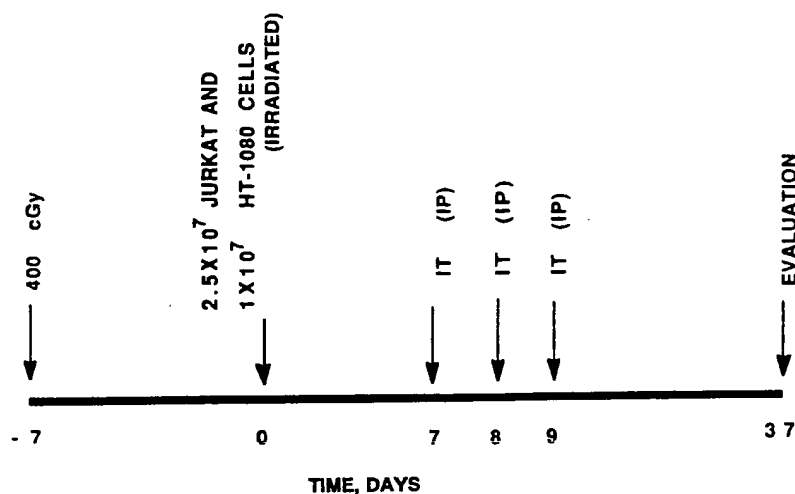


FIG. 2. Nude mice were preconditioned with 400 cGy of whole-body ^{137}Cs γ radiation on day -7. On day 0, 2.5×10^7 Jurkat cells (human T-cell leukemia, $\text{CD3}^+\text{CD4}^+\text{CD5}^+$) were injected subcutaneously with 10^7 HT-1080 feeder cells (human sarcoma) that had received 6000 cGy. Immunotoxin (IT) treatment was given by intraperitoneal (IP) injection starting on day 7 (when the tumor was visibly established) and was set at half the MLD in guinea pigs, which, like humans, are highly sensitive to DT. Mice were evaluated for the presence of visible tumor on day 37. See Table 1 and Fig. 1.

tent with our view that CD3 is routed optimally whereas CD5 is not. The cleavable crosslinker conferred no therapeutic advantage to anti-CD3-CRM9 immunotoxins and may have been less effective (group 3). Cleavable crosslinkers confer some advantage with anti-CD5-CRM9 conjugates *in vitro* (10) but had no effect in this *in vivo* system (group 5) and lacked significant potentiating effect when administered with anti-CD3-CRM9 (group 2). (The cleavable crosslinker conferred a marked therapeutic advantage with anti-CD5 wild-type toxin conjugates, and 80% tumor regressions were achieved with a single 6- μg dose; however, this amount exceeded the guinea pig toxic dose.)

In groups 7-9 (Table 1) increasing single doses of whole-body γ radiation were given to animals bearing $3 \times 3 \times 5\text{-mm}$ tumors. At 400 cGy, no complete regressions occurred. At 500 cGy, 50% complete tumor regressions occurred. At 600 cGy, 100% regressions was achieved as judged on the initial evaluation day, day 14. Evaluation on day 37 could not be performed because the animals died from radiation sickness

on days 17 and 20. (Groups 7-9 did not receive prior radiation and tumor takes were less than 100%.) It appears that the anti-CD3-CRM9 noncleavable immunotoxin at 75 $\mu\text{g}/\text{kg}$ is equal in therapeutic power to between 500 and 600 cGy of radiation.

The actual cell kills achieved by the radiation and by the immunotoxin can be compared by assuming single-hit inactivation kinetics along with a D_{50} value for the radiation. At 500 cGy we observed 50% survivors. This is the fraction of animals free of viable malignant cells and represents the zeroth term of the Poisson distribution $S = e^{-a}$, where a is the average number of viable tumor cells per animal (a equals 0.69 for this case and 0.22 for the case of 80% anti-CD3-CRM9 treatment survivors). The exponent, a , may be considered to be the product of two terms representing the pretreatment number of viable malignant cells, N_c , and the fraction of surviving tumor cells, $2^{-d/D_{50}}$, where D_{50} is the dose required to reduce the number of tumor cells by 50% and d is the delivered dose (17). A D_{50} value of 52 cGy is not

Table 1. CRM9-immunotoxin and radiation treatment on subcutaneous human T-cell tumors (Jurkat) in nude mice

Group	Treatment*	Mice with tumors at day 37, no./no. in group	% tumor regressions	$P'(x)^\dagger$
1	Anti-CD3-CRM9(NC) (25 $\mu\text{g}/\text{kg}$, $\times 3$)	1/6	83	6×10^{-7}
2	Anti-CD3-CRM9(NC) + anti-CD5-CRM9(C) (each at 19 $\mu\text{g}/\text{kg}$, $\times 2$)	1/4	75	2.6×10^{-4}
3	Anti-CD3-CRM9(C) (25 $\mu\text{g}/\text{kg}$, $\times 3$)	2/4	50	9.1×10^{-3}
4	Anti-CD3 + CRM9 (25 $\mu\text{g}/\text{kg}$, $\times 3$)	4/4	0	
5	Anti-CD5-CRM9(C) (25 $\mu\text{g}/\text{kg}$, $\times 3$)	5/5	0	
6	Anti-CD5-DT(NC) (25 $\mu\text{g}/\text{kg}$, $\times 1$)	9/9	0	
7	γ radiation (400 cGy)	2/2	0	
8	γ radiation (500 cGy)	3/6	50	1.1×10^{-3}
9	γ radiation (600 cGy)	0/2 [‡]	100	1.6×10^{-3}
10	None	6/6	0	

*Anti-CD3 refers to the monoclonal antibody UCHT1 (15) and was purchased from Oxoid USA, Columbia, MD. Anti-CD5 refers to the monoclonal antibody T101 (16) and was a gift from Hybritech. NC and C refer respectively to noncleavable and cleavable conjugates. Immunotoxins were administered intraperitoneally at the indicated doses on day 7 ($\times 1$), on days 7 and 8 ($\times 2$), or on days 7-9 ($\times 3$). Radiation was from a ^{137}Cs source.

[†]Maximum probability that the regressions observed within a group occurred by chance (see *Materials and Methods*).

[‡]These animals were evaluated on the initial evaluation day, day 14, rather than day 37 because of death due to radiation sickness on days 17 and 20.

unreasonable for a rapidly dividing helper T cell (18) and allows us to calculate N_c as 0.5×10^3 cells.[†]

This value of N_c permits determination of the fraction of surviving cells for the 80% survival immunotoxin group (0.4×10^{-3}) and determination of the radiation dose required to reach 80% survivors (590 cGy). This value is only approximate, since the estimate of 50% tumor-free survival was based on 6 mice. Radiation death of the animals prevented our observation of survivors at 600 cGy. However, we did determine the plating efficiency by limiting dilution of 7-day established Jurkat tumors (following dispersal) and tumors exposed earlier *in vivo* to 600 cGy. Plating efficiencies were 0.14 and 0.14×10^{-3} , respectively. The fractional cell survival was within a factor of 3 of the calculations based on a D_{50} of 52 cGy. We conclude that the immunotoxin is producing an approximate 3-log kill of Jurkat tumor cells *in vivo*.

DISCUSSION

These data show that anti-CD3-CRM9 can induce lasting regressions of established human T-cell tumors in a nude mouse xenograft system at a dose set at half the guinea pig MLD. The T-cell-killing power at this dose is equivalent to 500–600 cGy of γ radiation from a ^{137}Cs source. The *in vivo* data show that this binding-site-mutant holo-immunotoxin has a greatly enhanced therapeutic margin over wild-type toxin conjugates, which bears out previous expectations based on *in vitro* studies (7, 10). Even though the animal groups in this study are small, all of the anti-CD3-CRM9 treatment groups and the radiation treatment groups show a highly significant incidence of regressions compared with control groups with risk levels all <0.01 .

Eighty percent regressions of an established human xenograft tumor by an immunotoxin that does not crossreact with nontargeted tissues is almost unprecedented. Only one other report is known to us, the eradication of Reed–Sternberg tumors by anti-CD30-ricin A chain in nude mice (19). The high efficacy of the anti-Reed–Sternberg immunotoxin over other A-chain immunotoxins is very likely due to the very high number of receptors per cell [3×10^6 receptors (20) compared with 7×10^4 Jurkat CD3 receptors (21)]. The anti-CD3-CRM9 system, in contrast, relies on the maintenance of essential translocation and intracellular routing functions and achieves its therapeutic effect at 7% of the mole dosage used for ricin A-chain immunotoxins (19, 20, 22).

The applicability of CRM9-based immunotoxins directed at other epitopes and cell types is likely to increase as our knowledge of the biochemical basis of intracellular routing expands. Improvements in the therapeutic margin of this type of immunotoxin might be expected by engineering additional mutations into the C-terminal binding domain or by truncating this domain (23). The demarcation between the necessary translocation domain and the binding domain has not been precisely defined. However, the translocation domain clearly extends beyond the C terminus of CRM45 (residue 346) (24) and may extend to Cys-471 (25).

It should be emphasized that with high-affinity holo-immunotoxins (unlike conventional anti-neoplastic drugs) the fractional cell kill is inversely proportional to the target cell number (26, 27). To put this in perspective, the tumor burden in this study is almost equal to the number of T cells in a mouse ($\approx 10^6$). If extrapolation to humans holds, we estimate that a tolerated dose of anti-CD3-CRM9 immunotoxin could achieve *in vivo* a 3-log depletion of a normal number of CD3-positive T cells. Although we have not assessed the log kill of anti-CD3-CRM9 against human

peripheral T cells, the receptor number is similar to that in Jurkat cells (21). This suggests to us that this immunotoxin might be extremely useful in the treatment of immune-system disorders involving oligoclonal T-cell expansion without dramatic increases in total T-cell number. An example is graft-versus-host disease, a morbid complication of bone marrow transplantation. Marrow transplantation is often performed as anti-leukemia/lymphoma therapy. The T cells causing graft-versus-host disease are derived from the donor bone marrow. They appear to be necessary in the initial post-graft period to ensure marrow engraftment and to provide a graft-versus-leukemia effect (28, 29) but could be subsequently eliminated. An anti-human CD5-ricin A-chain immunotoxin has been used for this purpose with modest clinical success (30). However, this A-chain immunotoxin lacks the efficacy of anti-CD3-CRM9 when judged in a nude mouse xenograft system, as shown by the appearance of late tumor recurrences (22).

A second use for this immunotoxin is the experimental therapy of AIDS. Recently, Simard and Jolicoeur (31) reported the paradoxical finding that cyclophosphamide, an immunosuppressive reagent, protected mice from immunodeficiency initiated by the defective Duplain strain of murine leukemia virus. The protective effect appears to be mediated by ablation of an expanded clone of infected T cells that, by an unknown mechanism, produces immunodeficiency. There is one report of successful eradication of human immunodeficiency virus (HIV) infection by radiation induced T-cell ablation combined with high-dose zidovudine therapy followed by rescue bone marrow transplantation (32). Anti-CD3-CRM9 holds two therapeutic advantages over T-cell ablation induced by radiation or alkylating agents: (i) a much higher degree of T-cell specificity and (ii) the ability to kill nonactivated T cells because its toxic action inhibits protein synthesis rather than cell division (2). The latter could be most important, since HIV can enter resting peripheral lymphocytes and synthesize nonintegrative forms of viral DNA. These structures have been postulated as intermediate latent forms responsible for both latency and long-lived low-level persistence of HIV infection (33, 34).

Our speculations concerning the therapeutic utility of anti-CD3-CRM9 as a clinical agent for T-cell ablation are testable in nonhuman primates. A monoclonal antibody to a rhesus monkey CD3-like determinant, Rht3, has been developed (35). Whether anti-Rht3-CRM9-mediated T-cell ablation is associated with cytokine release and lymphoproliferative disorders, which plague current immunosuppressive regimes, could also be determined. In addition, rhesus models of autoimmune diseases (36) and simian AIDS (37) exist.

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[†]This number is considerably less than the number of cells within the tumor which exclude trypan blue, 4×10^7 , and reflects the low plating efficiency of this tumor in the nude mouse xenograft system.

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